EANES, LAUREN A., M.S. Analysis of Molecular Target(s) of Naringenin in MCF-7 Breast Cancer Cells (2014)
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Estrogen receptor (ER) antagonists such as tamoxifen have been used successfully to treat ER+ breast cancers for more than 30 years. Tamoxifen targets the ER and blocks the binding of estrogen thus preventing up-regulation of estrogen responsive genes and resulting in impaired cell proliferation. Unfortunately, long term use of tamoxifen can result in resistance. Previous studies have reported that naringenin, a flavanone, can inhibit cell proliferation and promote cell death in ER+ breast cancer cells. It has been reported that signaling pathways, such as mitogen activated protein kinase (MAPK), are up-regulated in tamoxifen resistant cells. Previous research suggests that naringenin inhibits the MAPK signaling pathways. For this study, I investigated the molecular targets of naringenin in tamoxifen-resistant cells in order to determine the mechanism of action of naringenin in impairing cell proliferation and survival to gain further insight in the mechanism of naringenin action and its use as a possible therapeutic treatment.

To determine if naringenin is targeting the MAPK pathway, tamoxifen resistant MCF-7 breast cancer cells were treated with naringenin or U0126, a MAPK kinase inhibitor. Our studies show that while both U0126 and naringenin impair cell proliferation and viability the combination of U0126 and naringenin resulted in greater inhibition of cell viability than either compound alone. It has been previously reported that naringenin can bind the ER. Our lab has also shown that

naringenin inhibits ERα from entering the nucleus thus ERα displays a peri-nuclear localization pattern. Confocal microscopy revealed that in U0126 treated cells ERα displayed an even distribution across the cell as seen in tamoxifen resistant cells. This suggests that MAPK is not the only target of naringenin. Thus to identify other possible molecular targets of naringenin we screened phage display libraries. Our phage display studies identified several members of the E3 ubiquitin ligase family which are responsible for targeting proteins for ubiquitination thus degradation. E3 ligases are important for proper regulation of protein levels within the cell. Together, our results suggest that MAPK is not the only target of naringenin. Instead these results suggest that naringenin could be targeting a more global protein such as E3 ligases and altering their activities thus altering the levels of target proteins involved in cell proliferation and survival. E3 ligases have been targeted by therapeutics for possible treatments in cancer and naringenin could be an effective therapeutic to target these E3 ligases.

ANALYSIS OF MOLECULAR TARGET(S) OF NARINGENIN IN MCF-7 BREAST CANCER CELLS

by

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Approved by

_____
Committee Chair

Dedicated to my wonderful husband, Ben, and my amazing family who inspire, love, and support me every day. All my thanks and praise is to God and to him be the glory!

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

Breast cancer affects more women than any other type of cancer. The American Cancer Society estimated breast cancer to be 29% (226,870) of all new cases of cancer among women with approximately 40,000 deaths in the year 2012 [1]. Cancer deaths reported from the years 1992-2008, ranked breast cancer first among women ages 20-59 years [1]. Current treatment involves surgery, chemotherapy, and endocrine therapy. Studies report early treatment with chemotherapy reduces odds of recurrence by 23% and death by 15%, while anti-hormone treatment with tamoxifen for 5 or more years reduces annual recurrence 40% and death by 30% in estrogen receptor (ER) positive cancer patients [2].

Estrogen and ER

Seventy percent of breast cancer patients are ER+ (most often ER alpha). Cell growth in these cells is stimulated primarily by estrogen [3, 4]. The ER is a nuclear hormone receptor and transcription factor. Transcription of ER-regulated genes is mediated by 2 domains: the ligand-independent activation factor 1 (AF-1) domain and the ligand-dependent activation factor 2 (AF-2) domain[3]. The ER is often localized within the nucleus, however it can be found in the cytoplasm and at the membrane [5, 6]. The presence of ERs in cancer cells determines treatment. Tumors

lacking ER respond infrequently to endocrine therapy, whereas response rates of 50 to 60 percent are observed in ER+ tumors [7].

Activation of ER

Activation of the ER can be achieved through ligand-dependent or independent pathways. Ligand-dependent activation of the ER depends on estrogen binding the ER. Following estrogen binding the ER forms a homodimer that translocates into the nucleus and binds to estrogen-responsive element (EREs) of target genes [8, 9].

In contrast, the ER can also induce a non-genomic rapid response [3, 6, 9, 10]. ERα can be found bound to the plasma membrane where the rapid, extra-nuclear response is initiated [6, 10]. Once bound by estrogen, the ER is released from the membrane initiating the activation of the mitogen-activated protein kinase (MAPK) pathway and the phosphoinositide 3-kinases (PI3K) pathways, both of which are pro-survival and growth [4, 6, 10]. ERα can also directly activate the epidermal growth factor receptor (EGFR), causing activation of the MAPK and PI3K pathways [4, 11, 12].

Ligand-independent activation of the ER (absence of E_2) is a result of phosphorylation of multiple serine and tyrosine residues in the AF-1 and AF-2 domains [3, 8, 13]. Growth factor receptors can activate the ER through several signaling pathways including the MAPK (Ras-Raf-MEK-ERK1/2) and PI3K(AKT) pathways [11, 13, 14]. ERK (Extracellular signal-regulated kinases) 1 and 2 and

AKT (protein kinase B or PKB) phosphorylate serine 118 of the ER resulting in its activation [5, 11]. Ligand-independent activation of the ER has increased the need for treatments that target not only the ER but also these signaling pathways to block cell growth.

ER and Breast Cancer

In ER+ MCF-7 breast cancer cells, binding of estrogen to the ER not only activates transcriptional regulation of genes involved in cell proliferation but also regulates genes involved in cell survival [15]. Estrogen responsive genes that are commonly upregulated in MCF-7 cells are Bcl-2, cyclin D-1, and survivin, all of which play a role in regulating cell proliferation and apoptosis [15].

The majority of breast cancer cells are dependent on estrogen stimulated growth which is why anti-estrogen treatments such as tamoxifen are so widely used [3, 5]. The most widely used anti-estrogens are the selective ER modulators (SERMs) [3]. SERMs allow for optimal function in all tissues where they function as estrogens in bone and cardiovascular tissues while functioning as anti-estrogens in breast and uterine tissue [3]. However, as previously stated the ER does not have to bind estradiol in order to become active. In the absence of estrogen (in the case of anti-estrogen treatments where the ER is blocked, or in mutated ERs) growth factor signaling pathways such as MAPK and PI3K activate the ER.

Tamoxifen

The most widely studied and used SERM is tamoxifen, which has been in use since the early 1970s [16, 17]. Tamoxifen has been proven to be a safe and effective treatment for advanced breast cancer [16, 18]. Tamoxifen binds the estrogen receptor, preventing estrogens from binding, thus inhibiting the expression of estrogen-regulated genes, and blocking the G1 phase of the cell cycle causing a decrease in cell proliferation [16, 19]. Studies indicate that the optimal tamoxifen treatment duration needed to decrease reoccurrence and improve survival is 5 years [16–19]. Unfortunately, the therapeutic benefits of tamoxifen are limited by acquired resistance [14, 20, 21]. Since multiple signaling pathways, such as the MAPK pathway, activate the ER, tamoxifen-resistant (Tam-R) cells have a heightened sensitivity to epidermal growth factor (EGF) and estradiol activation of MAPK as well as increased cross talk in proliferation pathways [20, 22, 23].

Activation of MAPK Signaling Pathway

ER+ Tam-R cell lines are reported to have constitutive activation of both the PI3K/AKT and MAPK pathways [4]. The MAPK cascades are important regulatory signaling pathways for cell proliferation, survival, and differentiation [24, 25]. The MAPK pathway is often mutated in various cascade kinase proteins, including Ras (the most frequently mutated oncogene), often leading to over-expressed or constitutively active signaling proteins [24]. Abnormal activation of the MAPK pathway can result in alterations of controlled cell growth and proliferation as well

as survival and migration, which in cancer cells creates a growth advantage, metastasis, and is often associated with therapy resistance [26, 27].

Naringenin

Naringenin is part of a large family of compounds found in fruits, vegetables, and many other plants [28–30]. Naringenin belongs to a group of natural substances known as flavanones. It has been shown that naringenin has an inhibitory effect on P450s, enzymes involved in biotransformation of various xenobiotics. Naringenin, along with other flavanoids, are linked to protective outcomes in regards to human health. For instance, flavanoids have been shown to reduce or protect against the development of endocrine tumors by binding the ER in individuals consuming a diet rich in flavanoids [6]. Furthermore, naringenin has been reported to induce apoptosis in different cancer cell lines containing ERα or ERβ (e.g., colon, breast, and uterus cancer cell lines)[6, 10].

More importantly naringenin, like other natural and synthetic substances, has been identified as an endocrine disruptor (ED) and more specifically a xenoestrogen. Compounds within the category of EDs and xenoestrogens, such as naringenin, Bisphenol A (BPA) and quercetin, are able to bind to steroid receptors [6]. Many EDs often have structures that mimic natural steroid hormones (e.g., E2) and enable EDs to interact with ERs as agonists or antagonists [6]. The steroid like structure also allows the substance to easily move through cell membranes because it is lipophilic.

Some flavanones alter hormone production and inhibit estrogen synthetase, a cytochrome p450 enzyme responsible for converting androgens to estrogens, such as estradiol, which have a major effect in the development of breast cancer [28, 30]. Naringenin has been shown to reduce glucose uptake and cell proliferation in MCF-7 breast cancer cells [28, 29]. The exact mechanism of reduced proliferation and growth arrest of the cells is not understood. Our previous findings suggest that naringenin affects the MAPK signaling pathway which can result in decreased proliferation and cell survival [10]. It has also been reported that naringenin induces apoptosis in various cancer cells including MCF-7 breast cancer cells [10, 31, 32].

Apoptosis

Apoptosis, programmed cell death, is a pathway that is stimulated by various stimuli and is regulated by caspases (cysteine aspartyl-specific proteases). Apoptosis is responsible for eliminating cells that have acquired excessive genomic damage, activated oncogenes, and cells that have progressed to malignancy [33]. Apoptosis can be promoted in response to ligands binding death receptors (intrinsic pathway) or by chemical/radiation damage (extrinsic pathway). Intrinsic apoptotic pathways lead to the activation of the initiator caspase 8. Extrinsic apoptotic pathways cause loss of mitochondrial stability and the release of Cytochrome C, a central player in apoptosis [34, 35]. Cytochrome C, once released into the cytosol, leads to the activation of intiator caspase 9, by cleaving the caspase. Active inititator

caspases lead to the cleaving of executioner caspases, such as caspases 3, 6, and 7. Active executioner caspases eventually cleave important cellular components, such as Poly ADP-ribose polymerase (PARP), resulting in cell degradation without inflammatory responses [34–36].

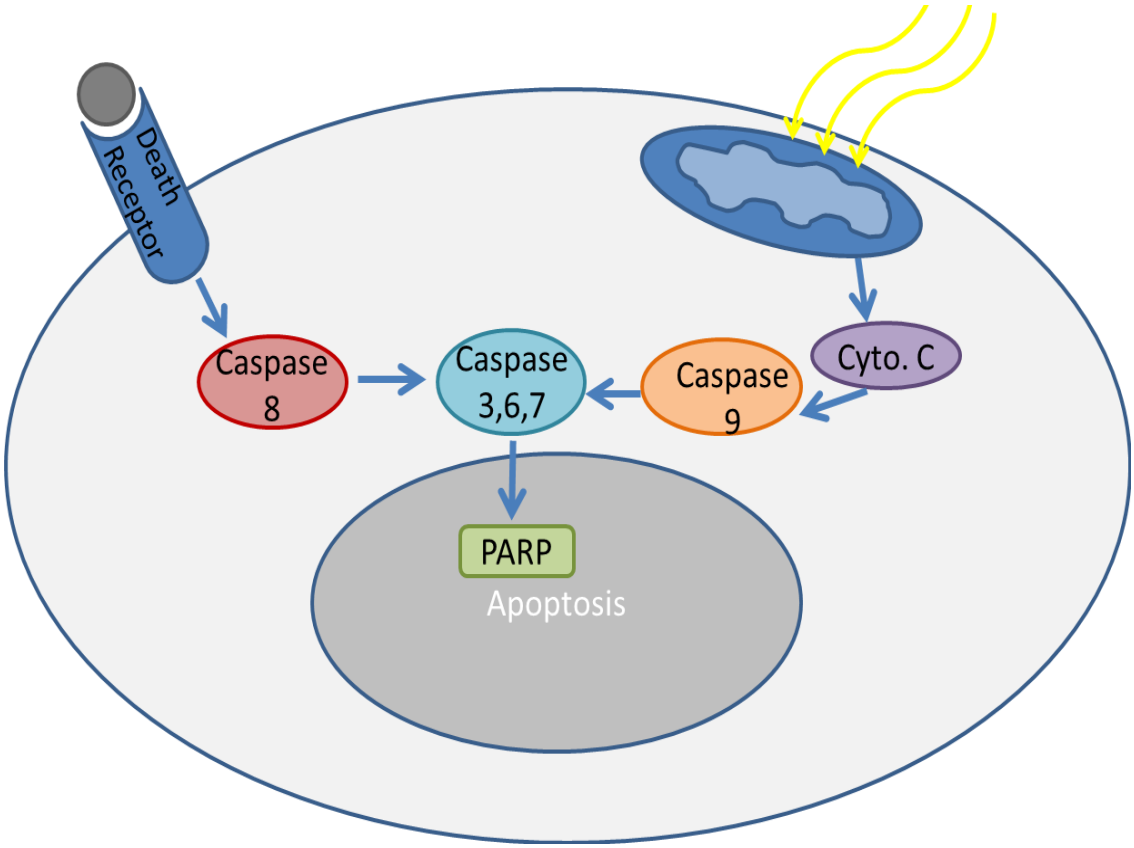


Figure 1. Overview of Apoptotic Pathways. Apoptosis is initiated through the activation of death receptors leading to the activation of caspase 8 or through DNA damage, exposure to chemical, or radiation causing the release of cytochrome C from the mitochondria, thus activating inititator caspases 9. The cleaving of inititator caspases 8 or 9 leads to the cleaving and thus activation of effector/executioner caspases 3,6, or 7 which then activates proteins such as PARP.

Naringenin and E3 Ligases

Currently E3 ubiquitin ligases, a large family of proteins responsible for the regulation of many cellular proteins and thus biological processes, have been targeted by therapeutics [37, 38]. E3 ligases catalyze the ubiquitination of proteins targeted for degradation. E3 ligases regulate a variety of proteins involved in cell proliferation, cell survival, DNA repair and cell signaling [39, 40]. It has been reported that E3 ligases are often overexpressed and abnormally regulated in various cancers thus leading to abnormal regulation/degradation of cellular proteins and ultimately cancer development [37]. E3 ligases can act as oncogenes (XIAP, MDM2 and HUWE1) or tumor suppressors (BRAC1, Fbw7, and CHIP) depending on the type of ligase and cell type [39].

Naringenin and MCF-7 Cells

Our lab has generated a tamoxifen resistant (Tam-R) MCF-7 cell line. This cell line, unlike the tamoxifen sensitive MCF-7 cell line, continues to grow in the presence of tamoxifen (measured by flow cytometry). The addition of naringenin has been shown to reverse proliferation in the Tam-R cells. Furthermore, the Tam-R cells exhibited an up-regulation of the MAPK pathway which could also be reversed with naringenin.

We also found that in tamoxifen resistant cells ERα exhibits a change in localization when compared to the tamoxifen sensitive MCF-7 cell line. ERα is distributed throughout the cell in MCF-7 cells (tamoxifen sensitive) and is found in

the nucleus when treated with tamoxifen. The Tam-R cell line has an even distribution of ERα across the cell similar to that found in untreated MCF-7 cells. However, upon treatment of the Tam-R cells with naringenin, the ERα localization becomes peri-nuclear. This suggests that naringenin is capable of changing the localization of ERα. Furthermore, naringenin and tamoxifen may work synergistically in Tam-R cells and could be even more effective in tamoxifen sensitive MCF-7 cells. The goal of this study is to identify the possible targets of naringenin in order to gain a greater understanding of the mechanism(s) involved in how naringenin affects MCF-7 cells proliferation and survival.

CHAPTER II

MATERIALS AND METHODS

Materials

MCF-7 ER+ breast cancer cells (HTB-22) were purchased from ATCC. Dulbecco's Modified Eagle Medium was purchased from Gibco (11885). Charcoal-stripped fetal bovine serum (F6765), naringenin (N5893) and 4-OH-tamoxifen (H7904) was purchased from Sigma Aldrich. Antibodies for ERK 1/2 (9102), p-ERK 1/2 (9101S), AKT (9272), p-AKT (4060S), Caspase 7 (9492P), PARP (9542P) and U0126 (9903) were purchased from Cell Signaling. Guava Via-Count Reagent (4000-040) was purchased from Millipore. Actin antibody (ab3280) was obtained from Abcam. Anti-ERα antibody (HC-20) was purchased from Santa Cruz biotechnology. AlexaFluor 488 conjugated Goat anti-Rabbit secondary antibody (111-545-003) was obtained from Jackson ImmunoResearch. Anti-mouse (A-4416) and anti-rabbit (A-6154) horseradish peroxidase conjugated secondary antibodies were purchased from Sigma Aldrich. The enhanced chemiluminescence (ECL) detection kit E-1119-20) was from BioExpress. Ph.D-7 Phage display peptide library (E8102L) was purchased from New England BioLabs. QIAprep spin M13 kit (27704) was purchased from Qiagen Sciences.

Cell Culture

Tam-R cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% charcoal-stripped FBS (Sigma Aldrich F6765) , 0.01 mg/mL bovine insulin, and 100 U/mL penicillin/streptomycin. Tam-R cells were maintained by adding 100nM of tamoxifen. Cells were maintained at 37° C and 5% CO_2. Media was replaced every two days and cells were passaged at 80% confluency.

Immunoblot

Protein extracts from various MCF-7 treatments were subjected to 10% SDS-PAGE and then transferred overnight to an Immobilon-P membrane (Millipore IPFL 00010). The membrane was incubated with the specific primary and secondary antibodies and visualized using enhanced chemiluminescent (ECL) and a Bio-Rad ChemiDoc XRS. The bands were quantified using densitometric analysis using Quantity One analysis software.

Flow Cytometry

MCF-7 cells were collected and centrifuged for 5 min at low speed (5), and the pellet was resuspended in 1x PBS. Guava Via-Count Reagent was added to cells at a 1:20 or 1:10 dilution and incubated for five min at room temperature in the dark. Guava easy-Cyte Flow Cytometry was used to determine the percentage of viable, mid-apoptotic, and dead cells. Values were analyzed by guavaSoft software.

Confocal/ ER Localization

MCF-7 cells were grown on cover slips. Cover slips were washed with 1xPBS twice. Cells were fixed with 3.7% paraformaldehyde for 15 min, washed twice with 1xPBS and then permeabilized for 5 min in Triton-X (0.25% in 1xPBS). Cover slips were blocked in a 5% goat serum (Jackson ImmunoResearch 005-000-121) - 1% BSA (MP, 81-00-33) - 1xPBS solution for 15 min. Cover slips were then incubated with anti-ERα primary antibody (1:100) for 1 h at room temperature. Cover slips were washed with 1xPBS then incubated with secondary antibodies (1:100 dilutions) for 45 min at room temperature. Cover slips were washed then stained with DAPI (1:1000) for 5 min. After the final washes, coverslips were mounted in one drop of Dako mounting medium (S3023) and viewed by confocal microscopy.

Phage Display

A random combinational constrained seven amino acid library (Ph.D.-C7C™) was propagated in *E. coli* ER2738 cells. Naringenin was immobilized to a plate with 0.1M $NaHCO_3$ (pH 8.6) overnight at 4^0C in a humidified container. A 100-fold dilution of phage (approximately $2x10^{11}$) were added to the plate, incubated and washed with TBST (TBS + 0.1% [v/v] Tween-20). After washing, the bound phage was eluted with 5% naringenin. Eluted phage was amplified by infecting ER2738 bacterial cells and subjected to three more rounds of selection on an immobilized naringenin plate. The fourth eluate was plated with ER2738 on LB plates. Twelve random colonies were picked and purified using a Qiagen Phage mini prep kit

before sequencing. The same procedures were carried out for a twelve amino acid library (Ph.D.-12).

CHAPTER III

RESULTS

Previous studies in our lab have shown that naringenin inhibits cell proliferation as a result of decreased cell viability. It has also been shown in our lab that naringenin inhibits ERK1/2 expression and phosphorylation and ERα translocation from the cytosol into the nucleus. These findings suggest that naringenin's effects on cell proliferation, viability, and ERα localization could be a result of inhibition of ERK1/2 phosphorylation and expression. In this study I wanted to determine if the inhibition of ERK1/2 could account for all of naringenin's effects on MCF-7 breast cancer cells.

Naringenin is a Weak Inhibitor of ERK1/2

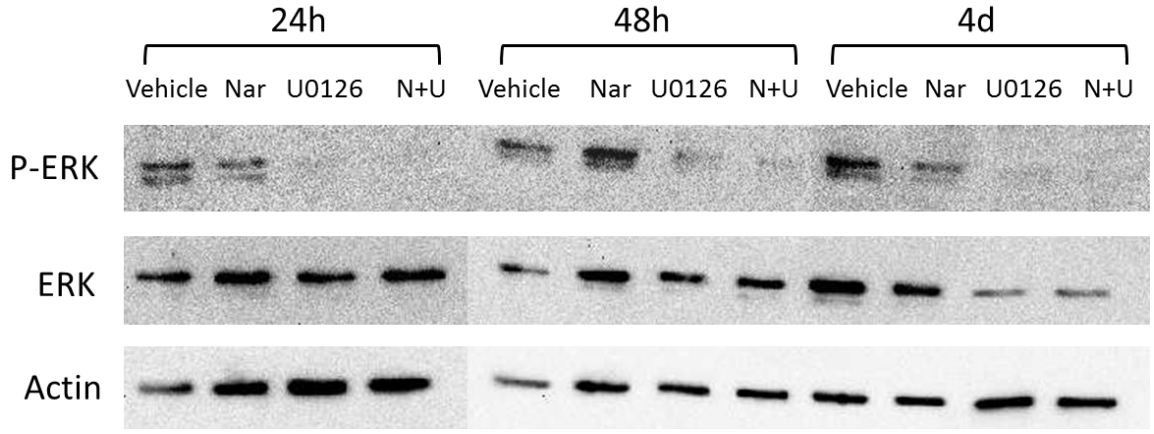
This study aims to determine if ERK1/2 is the primary target of naringenin in MCF-7 cells. I wanted to determine if the previously observed effects of naringenin in MCF-7 breast cancer cells could be accounted for by the inhibition of ERK1/2 phosphorylation. It has already been shown that naringenin inhibits the phosphorylation of ERK1/2 in charcoal-stripped medium. While charcoal-stripping of serum removes estrogen, it also removes other lipophilic compounds as well as growth factors that are involved in promoting cell proliferation and survival. Therefore, the use of charcoal-stripped medium allowed us to study the effects of

naringenin in an estrogen independent environment. We used Tam-R MCF-7 cells which have estrogen independent cell proliferation.

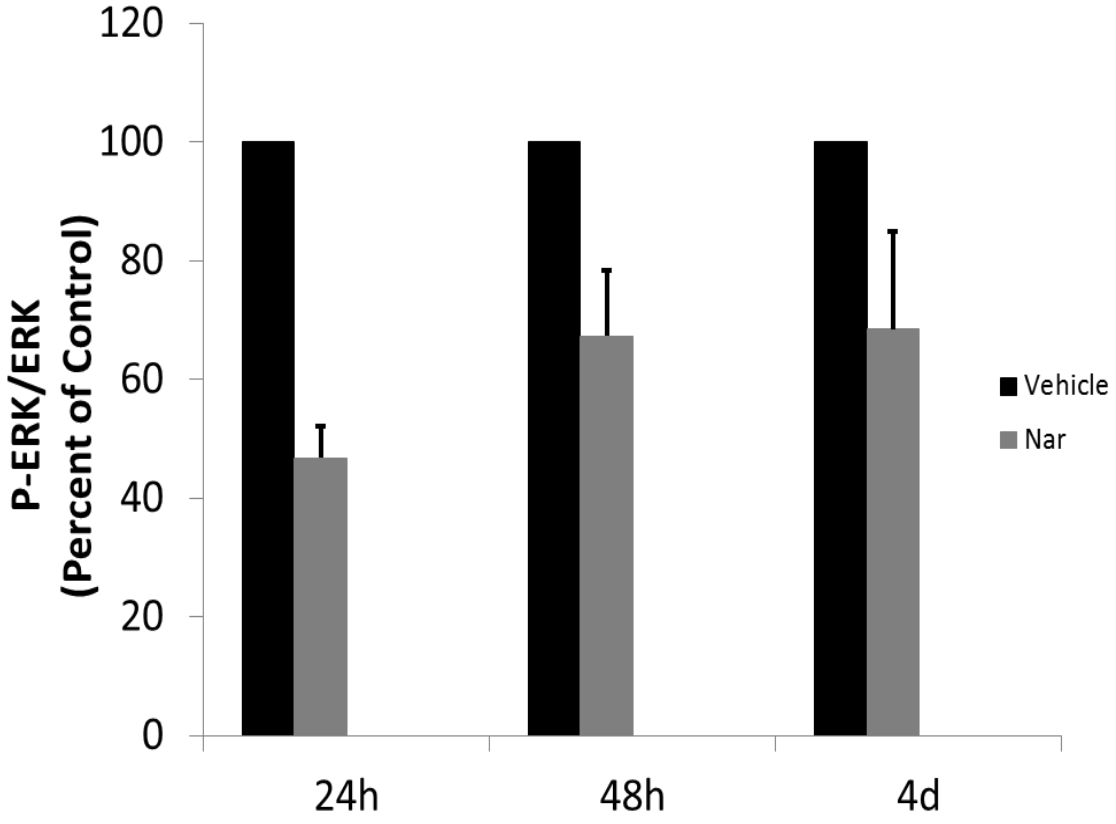
We compared the effects of naringenin and U0126, a known inhibitor of ERK1/2 phosphorylation on ERK1/2 expression and phosphorylation. To determine if U0126 elicits the same effect as naringenin on ERK1/2, we treated tamoxifen resistant MCF-7 cells with naringenin, U0126, and a combination of both for 24 h, 48 h, and 4 days.

The expression level and phosphorylation status of ERK1/2 were assayed using immunoblot analysis. The protein bands were quantified and normalized to the control group (untreated tamoxifen resistant MCF-7 cells). Our data confirmed that naringenin is an inhibitor of ERK1/2 phosphorylation at all the time points (Fig 2A). However, when compared to the U0126 treatment, naringenin appears to be a weak inhibitor of ERK1/2 (Fig 2A). Phosphorylated ERK1/2 was undetectable in the U0126 alone and the combination treatment at all time points thus U0126 significantly impaired the phosphorylation of ERK1/2 after 24 h and maintained this effect when compared to the control (Fig 2A and B). In contrast, naringenin treatment only resulted in a 50% inhibition of ERK1/2 phosphorylation at all reported time points. Lastly, all three treatments show a decrease in the amount of ERK after 4 days of treatment when compared to the control. However, this observed decrease is greater in the U0126 alone and combination treatments (Fig. 2A and C).

A.


24h
48h
4d
Vehicle Nar U0126 N+U
Vehicle Nar U0126 N+U
Vehicle Nar U0126 N+U
P-ERK
ERK
Actin

B.


120
100
80
60
40
20
0
P-ERK/ERK
(Percent of Control)
24h
48h
4d
Vehicle
Nar

C.

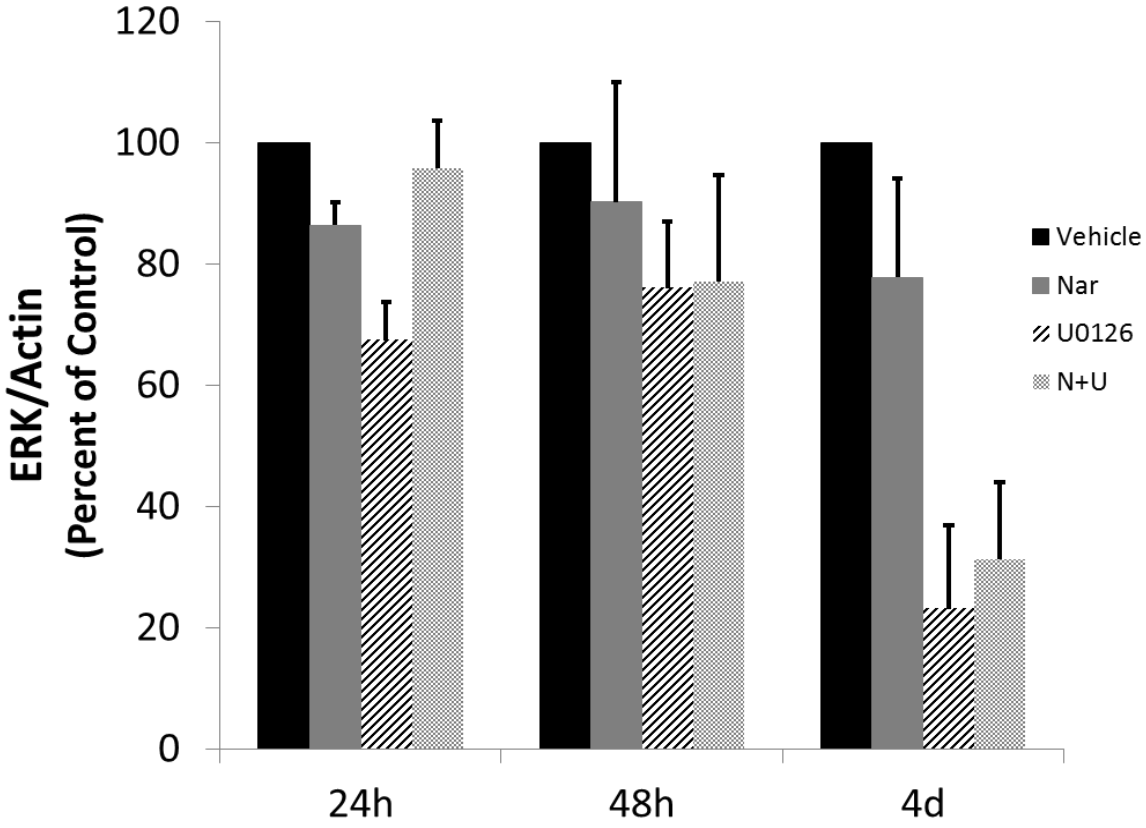


Figure 2. Naringenin is a Weak Inhibitor of ERK1/2 Phosphorylation. Tamoxifen resistant MCF-7 cells were grown in charcoal-stripped medium with 4-OHT (100 nM) in the presence of Nar (200 μM), U0126 (10 μM) or a combination of the two for 24 h, 48 h, and 4 days. A. Protein lysates were prepared. Lysates were subjected to SDS-PAGE and immunoblotted using antibodies against phospho-ERK 1/2, ERK 1/2 and actin. B. P-ERK to actin and C. ERK to actin were quantified using densitometric analysis by Quantity One software and are expressed as a percent of the control. The results are representative of 3 separate experiments.

Naringenin and Inhibition of ERK1/2 Decreased Cell Proliferation

Our lab as well as previous studies have reported that naringenin decreases cell proliferation [28, 30, 41]. This decrease in cell proliferation may be in part attributed to the observed inhibition on ERK1/2 expression and phosphorylation. ERK is a kinase involved in the MAPK pathway which promotes cell proliferation.

We wanted to determine if decreased cell proliferation is a direct result of ERK1/2 inhibition in naringenin treated cells. Tamoxifen resistant MCF-7 cells were treated as previously stated with naringenin, U0126, or a combination of the two and assayed for cell proliferation. The effects of U0126 on cell proliferation were compared to those of naringenin.

Cells from each treatment were prepared and cell density (cells/mL) was analyzed by flow cytometry. There was no significant difference in cell density in any of the treatment groups after 24 and 48 h when compared to the control. However, after 4 days of treatment all three groups showed a decrease in cell density. The U0126 treated cells had a slightly greater decrease in cell proliferation when compared to the control. Both U0126 and naringenin appear to elicit a similar effect on cell proliferation (Fig. 3).

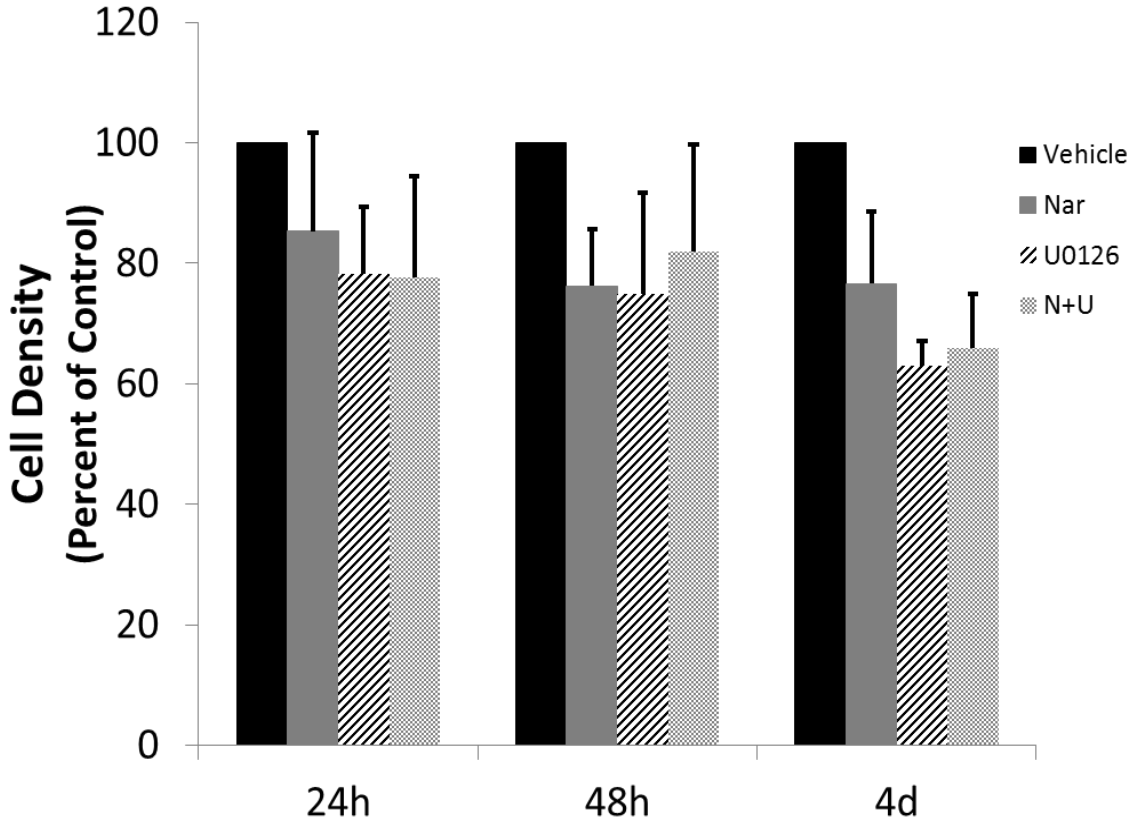


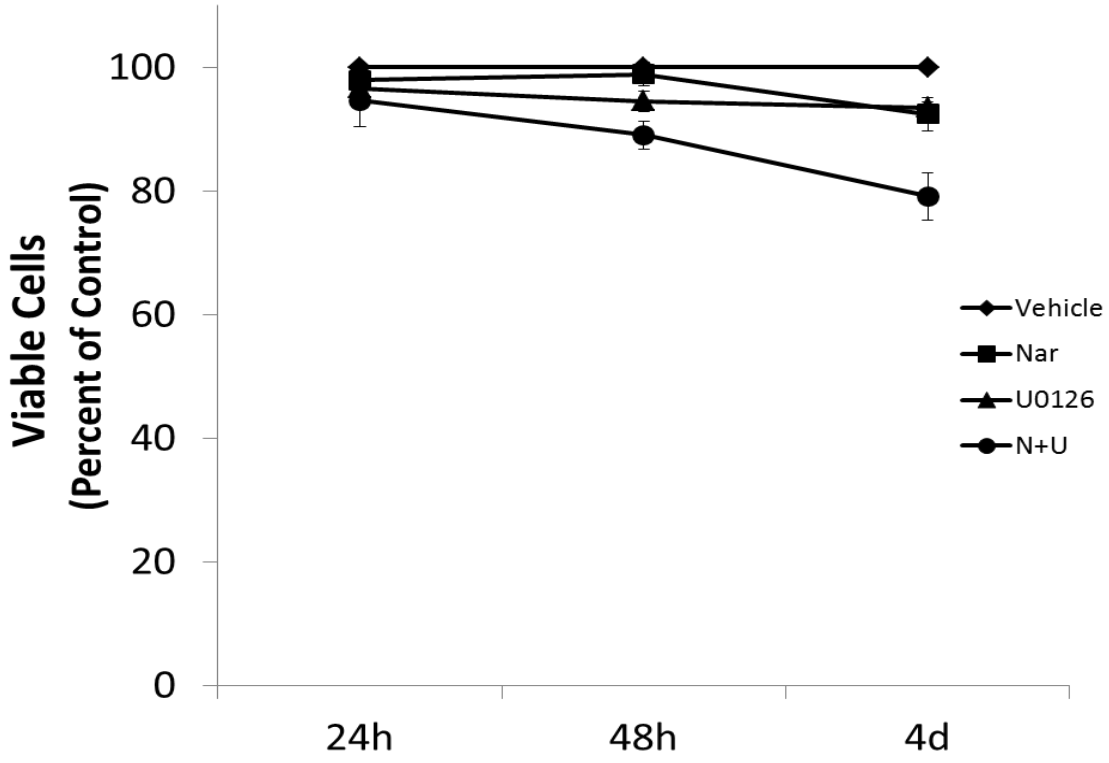
Figure 3. **Naringenin and U0126 Decrease Cell Density.** Tamoxifen resistant MCF-7 cells were grown in charcoal-stripped medium with 4-OHT (100 nM) in the presence of Nar (200 μM), U0126 (10 μM) or a combination of the two for 24 h, 48 h, or 4 days. Cell density (cells/mL) was determined by flow cytometry. Results are the means ± SEM of three separate experiments. Data were normalized to control.

Inhibition of ERK1/2 Alone Does Not Account for Decreased Viability

Naringenin has been reported to decrease cell proliferation as a result of decreased cell viability [31]. We wanted to determine if the effects on cell viability are a result of naringenin targeting and inhibiting ERK1/2. We treated tamoxifen resistant MCF-7 cells with U0126, naringenin, or a combination of the two as previously stated. Cells were collected from each treatment and prepared for flow cytometry analysis. Cell viability analysis revealed that both naringenin and U0126

reduced viability on day 4 to the same extent (Fig. 4A). However, when U0126 and naringenin were used in combination there appears to be an additive effect resulting in a greater decrease in cell viability. This same pattern was observed in cell death (Fig 4B).

A.



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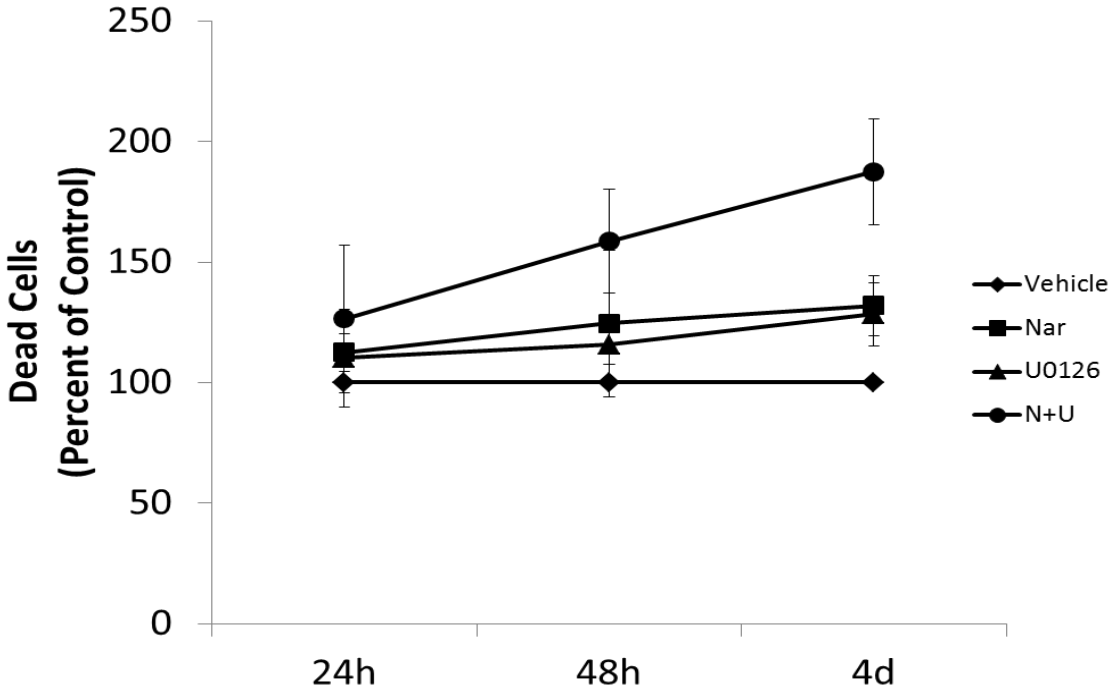


Figure 4. **Naringenin Does Not Inhibit Cell Viability by Inhibition of ERK.** Tamoxifen resistant MCF-7 cells were grown in charcoal-stripped medium with 4-OHT (100 nM) in the presence of Nar (200 μM), U0126 (10 μM) or a combination of the two for 24 h, 48 h, or 4 days. A Cell viability and B. cell death were determined by flow cytometry. Results are the means ± SEM of three independent experiments. Data were normalized to control.

Naringenin Induces Apoptosis

Previous studies reported that naringenin induces apoptosis through PARP and caspase activation in HeLa and MCF-7 cells [10, 31]. Our lab has shown that naringenin can induce apoptosis through the activation of caspase 7, which may explain the observed decrease in cell viability. In order to determine if induced apoptosis in naringenin treated cells is a result of ERK1/2 inhibition we examined the levels of apoptotic cells and the status of known apoptotic markers in U0126

treated cells. In this study we treated Tam-R MCF-7 cells with U0126, naringenin, or a combination of the two and determined the number of apoptotic cells to determine if the observed decrease in cell viability and proliferation correlated and whether inhibition of ERK1/2 alone was responsible for the effects of naringenin on viability.

Treated cells were prepared and analyzed by flow cytometry. Our findings show that there was an increase in apoptotic cells in all treatment groups when compared to the control (Fig. 5). Our data also indicated that there was no significant difference between the treatment groups over time. However, when compared to the control all three treatment groups show an increase in apoptotic cells beginning at 24 hours and continuing over each reported time point (Fig. 5). Our data for apoptotic cells correlates with the reported changes in cell viability and cell death where we saw a decrease in cell viability with a corresponding increase in cell death which can be explained by the increase in apoptosis.

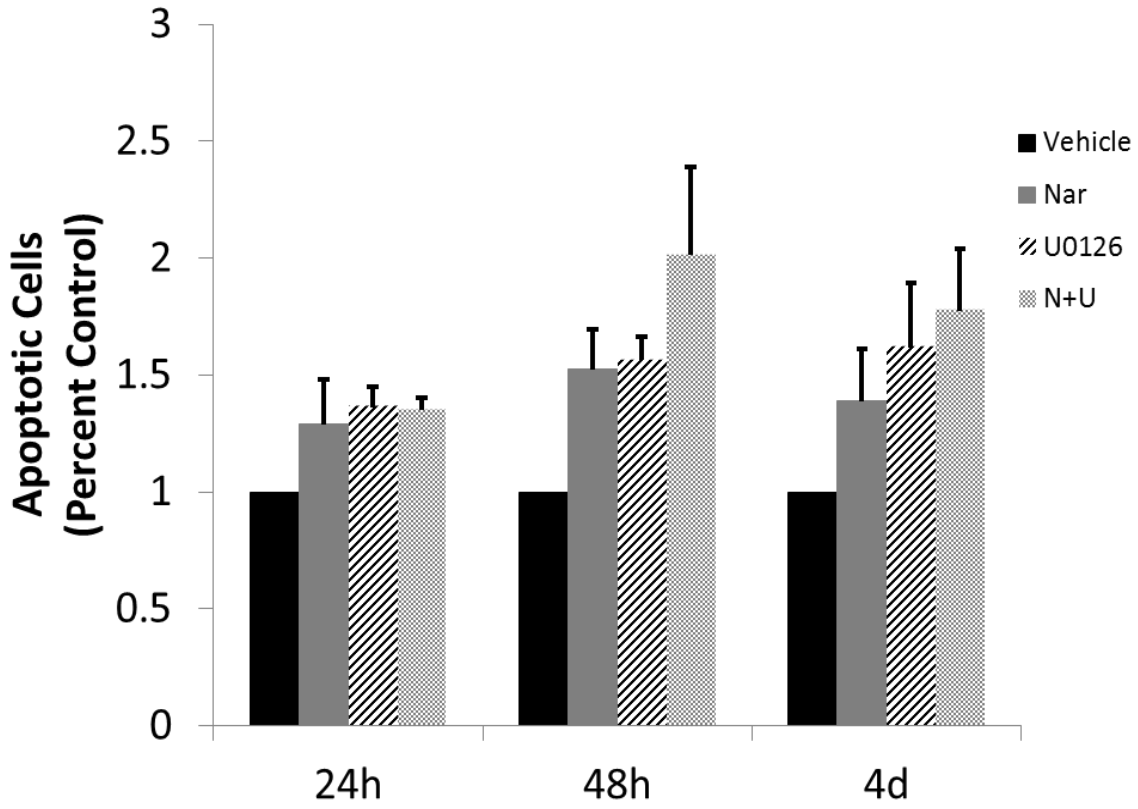


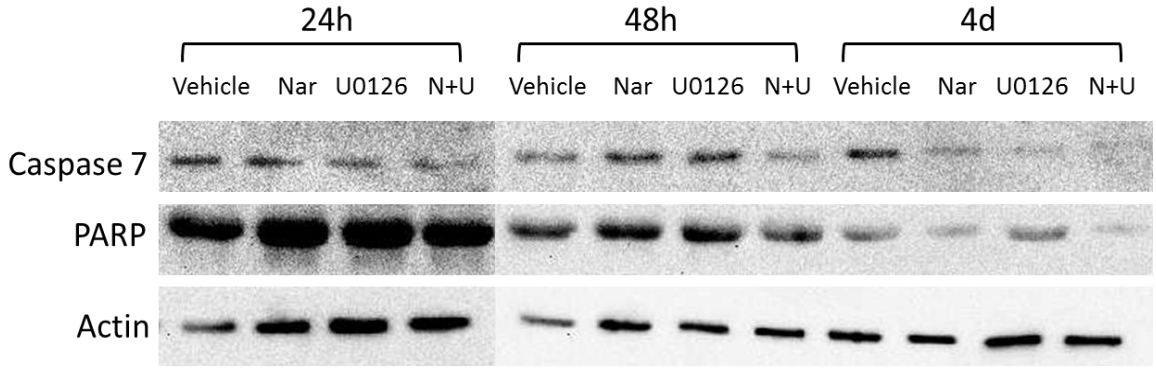
Figure 5. **Naringenin and U0126 Induce Apoptosis.** Tamoxifen resistant MCF-7 cells were grown in charcoal-stripped medium with 4-OHT (100 nM) in the presence of Nar (200 μM), U0126 (10 μM) or a combination of the two for 24 h, 48 h, or 4 days. Mid-apoptotic cells were determined by flow cytometry. Results are the means ± SEM of three independent experiments. Data were normalized to control.

To further analyze the mechanism of apoptosis we assayed the status of known apoptotic markers, caspase 7 and PARP, in naringenin and U0126 treated cells. Our results indicate that both naringenin and U0126 lead to the cleaving/activation of PARP and caspase 7. After 24 h all three treatments show a decrease in caspase 7 expression. Naringenin maintains an approximate 20% decrease in caspase 7 at all the time points. The combination treatment and U0126 alone show an almost complete activation of caspase 7 shown by the significant

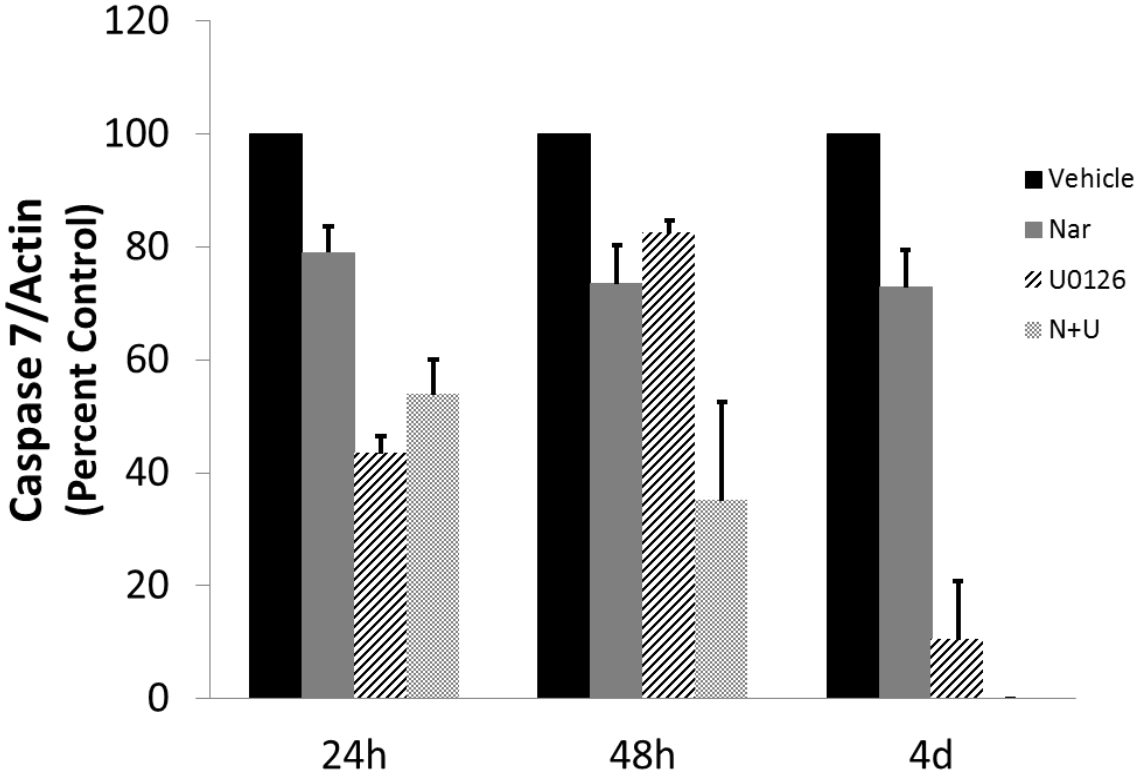
decrease to no detectable expression of caspase 7 at 4 days (Fig. 6A and B). The combination treatment shows a greater effect than naringenin or U0126 alone at 48h and 4 days (Fig. 6B)

Next we examined the expression of PARP which is downstream of the activated caspases. Quantified data for PARP expression shows all three treatments decrease PARP expression. Naringenin again maintains a 20% decrease in PARP while U0126 resulted in an approximate 30% decrease at all sampling times when compared to the control. The combination treatment shows an approximately 50% decrease across all three time points when compared to the control. Similar to the flow cytometry, the immunoblot analysis shows that the combination treatment has a greater effect than either naringenin or U0126 alone when compared to the control (Fig. 6). The combination treatment effect appears to be an additive effect when compared to either naringenin or U0126 alone.

A.


24h
48h
4d
Vehicle
Nar
U0126
N+U
Caspase 7
PARP
Actin

B.


Caspase 7/Actin
(Percent Control)
120
100
80
60
40
20
0
24h
48h
4d
Vehicle
Nar
U0126
N+U

C.

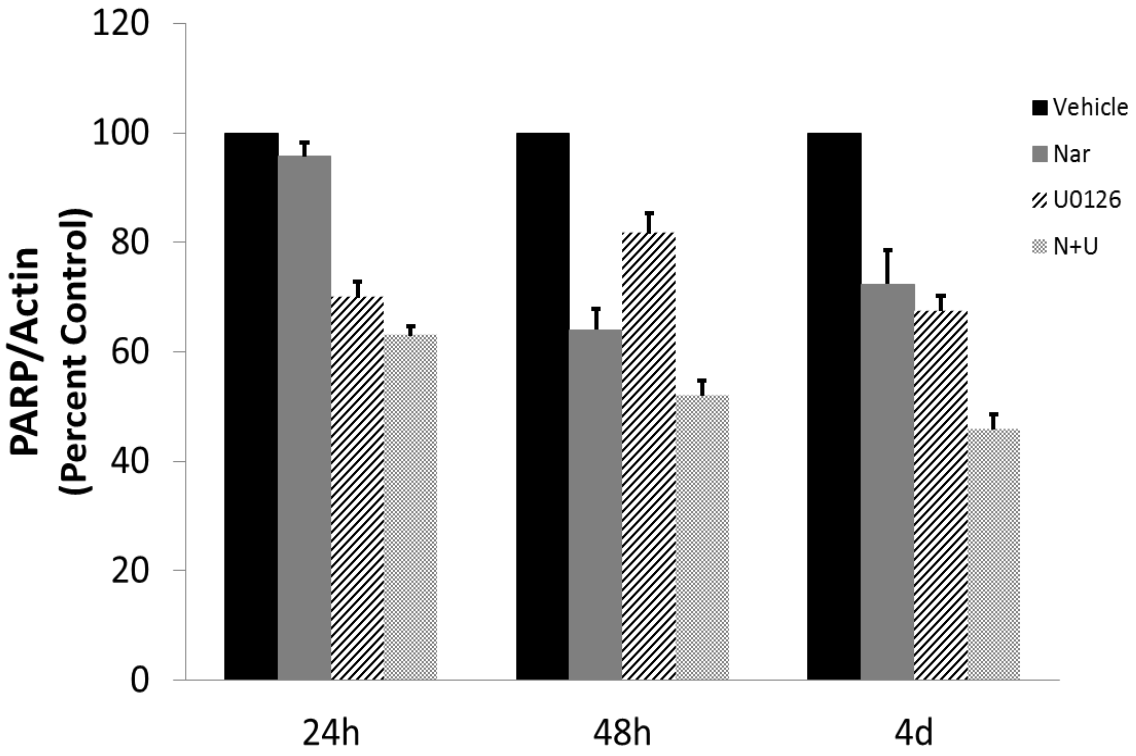


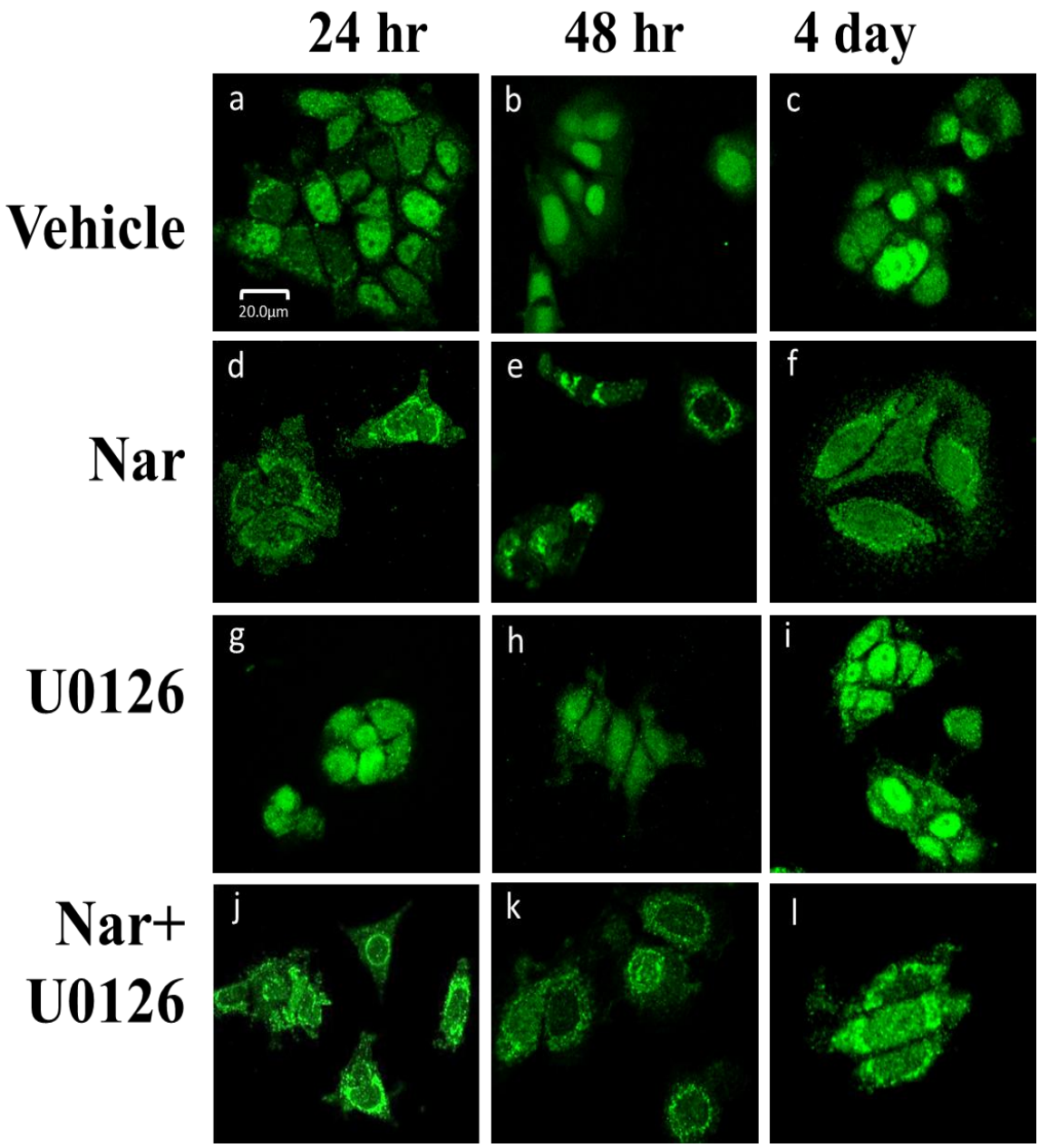
Figure 6. Naringenin and U0126 Decrease Expression of Apoptotic Markers. Tamoxifen resistant MCF-7 cells were grown in charcoal-stripped medium with 4-OHT (100 nM) in the presence of Nar (200 μM), U0126 (10 μM) or a combination of the two for 24 h, 48 h, and 4 days. A. Protein lysates were prepared and subjected to SDS-PAGE and immunoblotted using antibodies against Caspase 7, PARP, and actin. B. Caspase 7 and C. PARP were quantified using densitometric analysis using Quantity One software and are expressed as a percent of the control. Results are the means ± SEM of three separate experiments. Results are normalized to control.

Naringenin Alone Influences Estrogen Receptor Localization

Next we examined the localization pattern of ERα. ER is found in both the cytoplasm and nucleus [6, 42–44]. ERα can be activated and enter the nucleus through either the binding of a ligand (estrogen) or phosphorylation [6, 8, 9, 11–13]. We have shown that naringenin impairs cytoplasmic ERα from entering the nucleus.

To determine if the effects of naringenin on ERα localization were a result of naringenin inhibiting ERK1/2 we treated cells with U0126, naringenin, or a combination of the two as previously stated and performed confocal microscopy as described in methods. The cells were incubated with antibodies against ERα. Similar to previous studies, our results show that the control cells (tamoxifen resistant MCF-7) have an even distribution of ERα in the cytoplasm and nucleus in all three time points (Fig. 7A. a-c and B). U0126 treated cells also show an even distribution of ERα at all the time points with no difference compared to the control (Fig. 7A. g-i and B). In contrast, cells treated with naringenin and the combination treatment exhibited approximately 20-30% expression of ERα within the nucleus thus localizing more ERα within the cytoplasm compared to the control (Fig. 7 A. d-f; j-l and B). This data suggests that naringenin alone is responsible for the observed changes in ERα localization.

A.


24 hr
48 hr
4 day
Vehicle
Nar
U0126
Nar+
U0126
a
b
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d
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h
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k
l
20.0μm

B.

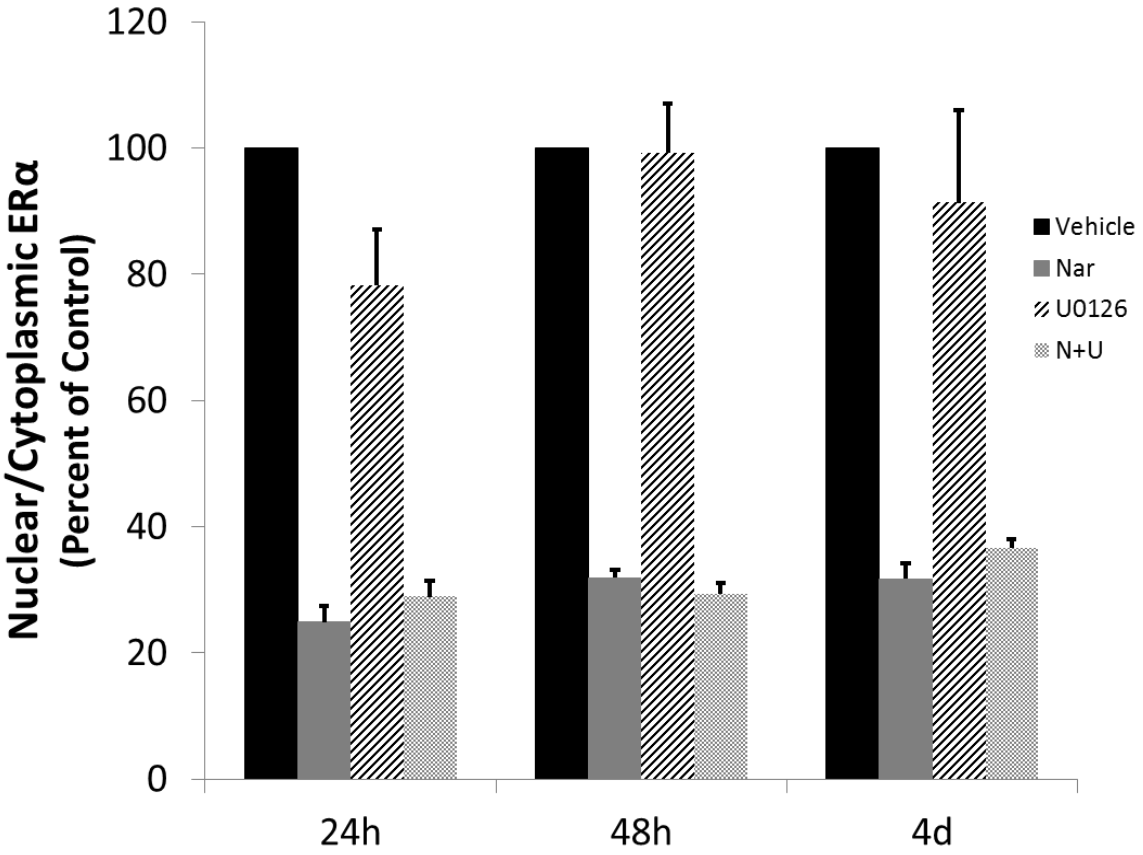

Figure 7. **Inhibition of ERK Cannot Account for ERα Localization Change.** Tamoxifen resistant MCF-7 cells were grown in charcoal-stripped medium with 4-OHT (100 nM) in the presence of Nar (200 μM), U0126 (10 μM) or a combination of the two for 24 h, 48 h, and 4 days. A. ERα localization was determined using confocal microscopy. B. Localization data was quantified using intensity parameters. The data is representative of three independent experiments.

Naringenin has Multiple Targets

Naringenin can interact with proteins involved in kinase signaling pathways and ERα [28, 30, 42–44]. We hypothesized that the effects of naringenin on cell proliferation, viability, and apoptotsis could be explained by interacting and inhibiting the MAPK pathway through ERK1/2. Thus far, our data suggest that the

effects of naringenin cannot be explained by ERK1/2 inhibition alone. Furthermore, our data confirmed naringenin changes ERα localization and this effect is specific to naringenin. Thus we aimed to identify other possible molecular targets that would account for the anti-proliferative and pro-apoptotic effects of naringenin.

To identify the targets of naringenin phage display libraries were screened. Phage display peptide libraries are randomly generated libraries in which 7 amino acid or 12 amino acid peptides are fused to the phage vector. We first had to immobilize naringenin to a surface. We coated 6cm plates with (100μg/mL) naringenin and tested the plates using mass spectrometry to determine whether naringenin bound to the plates. The analysis showed a strong naringenin (m/z 271) peak in the target solution that was added to the plate with an average signal of 7.64e3. After overnight incubation of the plate with the naringenin solution, the solution had an average signal of 5.17e3 naringenin. Lastly, the solution collected following multiple washes of the plate shows no detectable amount of naringenin which indicates that little to no naringenin was removed during the washing process. Although we are not able to directly detect naringenin on the plate, collectively, our findings suggest that naringenin bound the plates (Fig. 8).

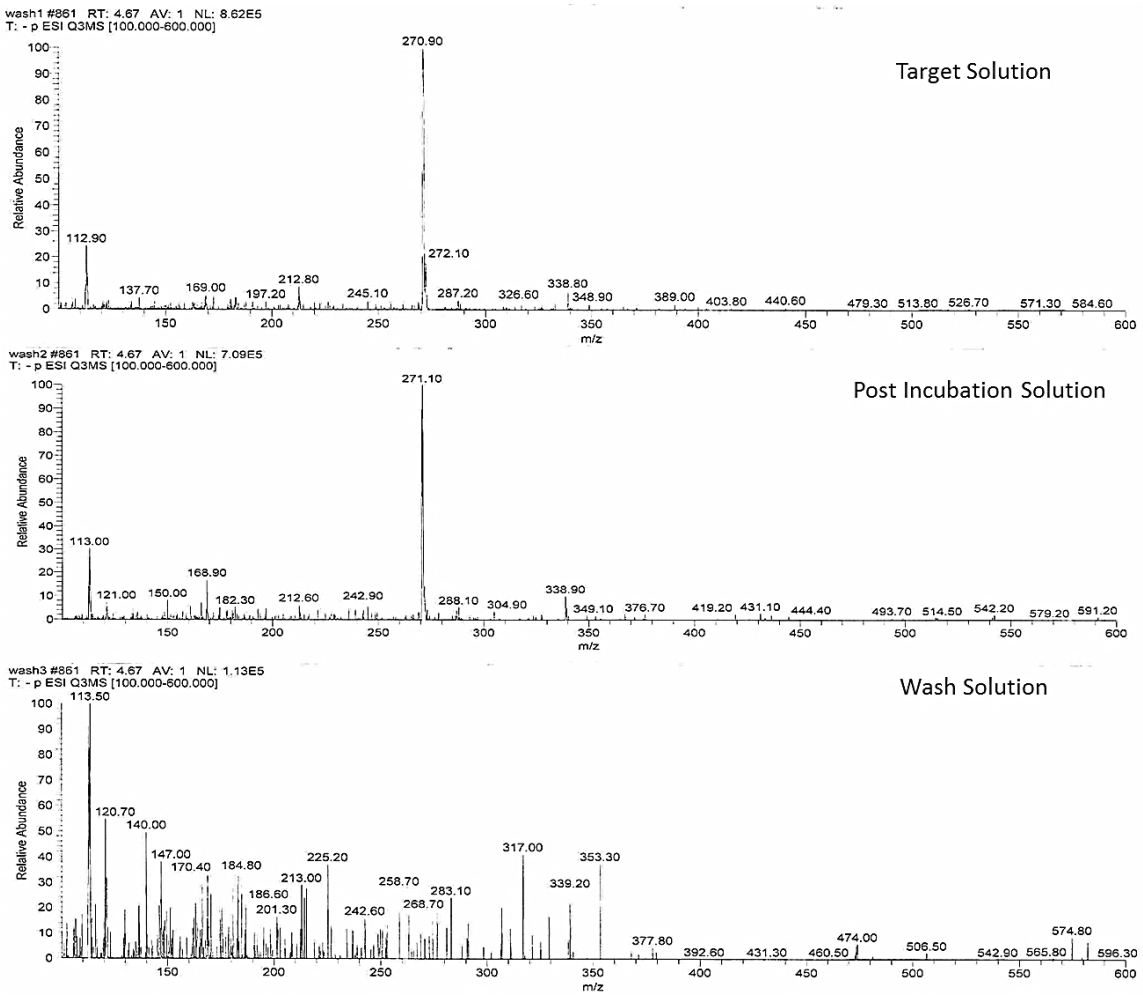

Figure 8. Naringenin Binds to the Plate. Mass Spectrometry analysis was performed for the naringenin target solution added to the plates, the post-incubation solution removed from the plates, and the solution removed from the plates following washes. The data is representative of three independent experiments.

While phage screening is a known procedure it is an underutilized procedure for the identification of molecular targets in an unbiased manner. Thus, there are no reported studies using phage screening to identify all the possible targets of naringenin. The process of identifying peptide sequences that bind to naringenin through the use of peptide-bound phage libraries requires several rounds of

selection and amplification of phage (Fig. 9). In our study we used both a 7 and 12 amino acid library to identify the targets of naringenin. Both the 7 and 12 libraries have densities on the order of 10^9 independent clones. The 7 amino acid library provides a full range of all possible combinations of amino acids but there is less binding affinity and specificity. The 12 amino acid library provides increased specificity and binding affinity but less range because it does not have all the possible combinations of amino acids. The use of both libraries allows us to compare the sequence data and harness the benefits of both libraries [45].

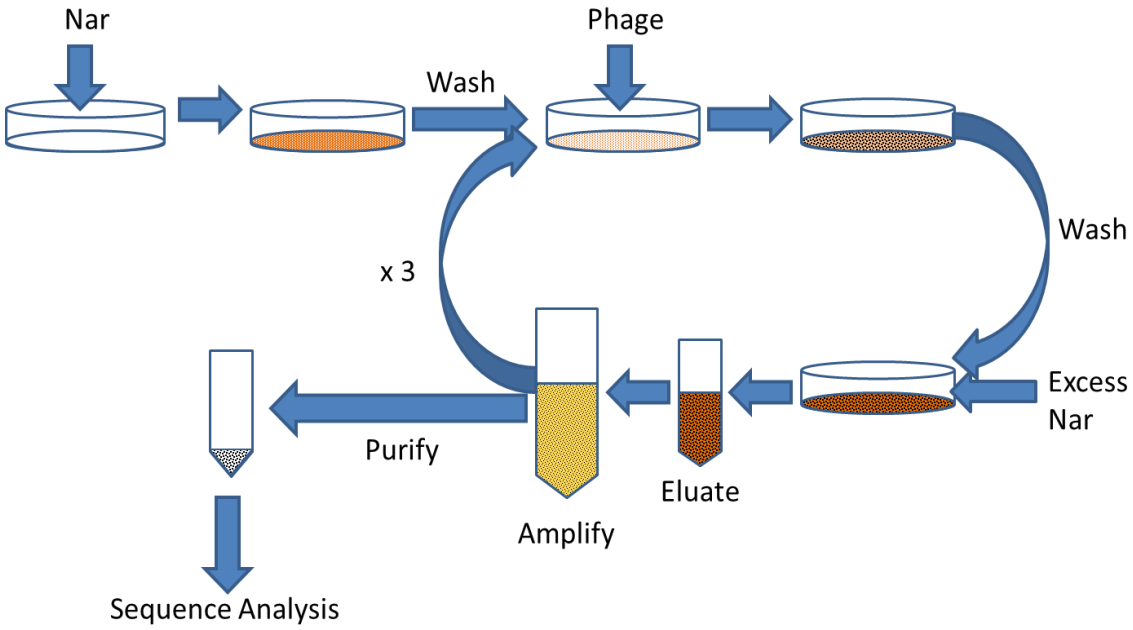

Figure 9. Overview of the Phage Screening Process. Naringenin was immobilized to the plates and washed before adding the desired phage library followed by incubation. The plates are washed before adding a naringenin solution to compete the bound phage creating the eluate. The eluate was amplified in ER2738 E.coli and the phage were isolated. The process was repeated 3 more times before selecting and purifying the phage for sequence analysis.

Following 4 rounds of amplification, phage were purified using a Qiagen prep kit and sequenced. There was no consensus among the identified sequences; however, there were a few repeated sequences such as HAIYPRH (Table 1). Our screen did not identify sequences reported by the manufacturer that are known to bind to plastic and are thus non-specific [46]. Our sequence data revealed that known targets of naringenin such as the estrogen receptor and cytochrome p450s were identified in our screen. Collectively, this provides further support that naringenin was immobilized to the plate and that this procedure was effective in identifying molecular targets of naringenin.

We identified multiple sequences of proteins involved in the ubiquitin pathway, specifically numerous E3 ubiquitin ligase enzymes. We also identified sequences present in several kinase proteins including proteins related to the MAPK signaling pathway, which was also expected because naringenin interacts with the MAPK pathway. Proteins involved in transcriptional regulation were also identified. Lastly, there were multiple proteins identified that contain zinc finger and coiled-coil domains (Table 2).

After further analysis of the identified proteins involved in the ubiquitin pathway we found that several of the E3 ligases are overexpressed in cancers such as breast cancer. E3 ubiquitin ligase XIAP is involved in antiapototic signaling by targeting caspases and is found to be overexpressed in cancers [39, 47]. F-box/WD repeat-containing protein 7 has been reported to be overexpressed in breast cancer and targets cyclin E [39]. Furthermore, E3 ubiquitin protein ligase HUWE1 is

overexpressed in breast cancer and targets p53 thus promoting cell growth and antiapoptosis signaling [39, 48] (Table 3). E3 ubiquitin-protein ligases Itchy and DTX1 have been reported as negative regulators of NOTCH 1, a protein involved in the Hippo signaling pathway which promotes cell proliferation and differentiation [49, 50]. Lastly, it is important to note that E3 ligase UBR5 has been reported to be disrupted in cancers (overexpressed in breast cancer) and is a regulator of ERα protein levels and transcriptional activity and thus cell proliferation [51].

Table 1. Identified Sequences from Phage Screening.

7-mer Library	12-mer Library
Y S I P K S S	L E S H Y T Q A S Y T Q (2)
A Q P A L Q R	K L H I S K D H I Y P T
E D L R W W T	H S T A G W L H S Y M I (2)
L P L T P L P (2)	N M E L H P H S L P R P
M V P K W V A	M A H T H T P S V T I T (2)
H A I Y P R H (3)	L V F P V T S D T I L F
G H W A A L V	
W Q T S P P F	
H E K P T R H	
W E A H A P L	
G P M L A R G	
W P T L Q W A	
G N T P S R A	
L P L Y I K S	
K I P I A L S	
N N T M M H H	
T F I N P S H	
S S F P P L L	

(#)-indicates a sequence that was selected more than once

Table 2. Most Frequent Categories of Proteins from Phage Screening.

Families of Proteins	Examples
Ubiquitin Pathway	E3 ubiquitin-protein ligase UBR3 UBR5 HUWE1 XIAP Itchy DTX1 DTX4 TRIM23 F-box/LRR-repeat protein 13 F-box/WD repeat-containing protein 7 F-box/WD repeat-containing protein 10 Ubiquitin-conjugating enzyme E2 O
Kinase	MAP4K4 MAP3K7 MAPK7 serine/threonine-protein kinase LATS1 PLK1 OSR1 kinase suppressor of Ras 2 (2)
Receptors	G-protein coupled receptor 1 and 98 Steroid hormone receptor ERR1
Zinc Finger	Zinc finger protein 30, 266, 423 , 404, 526 (2), 808, 462
Transcription Regulation	Nuclear receptor corepressor 1 Nuclear receptor coactivator 2 Transcription initiation factor TFIID transcriptional-regulating factor 1 Transcription factor COE1 Histone deacetylase complex subunit SAP130 Mediator of RNA polymerase II transcription RNA polymerase II-associated protein 1 Forkhead box protein P1, P2, I3
Drug Metabolism	Cytochrome P450 1A2 2R1 26C1 2W1
Coiled-Coil	Coiled-coil domain-containing protein 84, 141, 142(2)

Table 3. Ubiquitin Pathway Proteins with Correlating Domains and Function.

Class	Sequence	Protein	Domain	Function
Ring	N M E L H P H S L P R P	E3 ub-protein ligase DTX1 & DTX4	Directly downstream Ring domain and in DTX domain	Ubiquitination of MEKK1, NOTCH 1 regulation
	Y S I P K S S	E3 ub-protein ligase ZNRF2	100 bp upstream Ring domain	
		E3 ub-protein ligase DTX1	~15 bp downstream WWE domain	Ubiquitination of MEKK1, NOTCH 1 regulation
	H A I Y P R H	E3 ub-protein ligase XIAP	BIR domain-inhibits caspases thus apoptosis	Overexpressed in cancers-Stops apoptotic cell death
F-box	H S T A G W L H S Y M I	F-box/WD repeat-containing protein 13	~100 bp Upstream F-box	
	M A H T H T P S V T I T	F-box/WD repeat-containing protein 7	~50 bp upstream F-box	Mutations in ovarian and breast cancer-ubiquitination of cyclin E
	G H W A A L V	F-box/WD repeat-containing protein 10	~100 bp upstream WD40 domain – protein-protein interaction	
UBR	K L H I S K D H I Y P T	E3 ub-protein ligase UBR3		Regulates APE1 required for DNA repair and genome stability
	E D L R W W T	E3 ub-protein ligase UBR5	N-terminal Ubquitin-associated domain	Disrupted in cancers-increased in breast cancer regulator of ERα protein levels and transcriptional activity thus cell proliferation

HECT	Y S I P K S S	E3 ub-protein ligase HUWE1	N-terminus end - domain of unknown function	Overexpressed in breast and colorectal cancer. Major target p53/TP53 tumor suppressor promotes cell growth and antiapoptosis
	H A I Y P R H	E3 ub-protein ligase Itchy	C-terminal HECT catalytic domain for E2 conjugation	Negative regulator of Large Tumor Suppressor 1 (LATS1) of Hippo Signaling Pathway-tumorogenesis, differentiation and renewal. Ubiquitination of NOTCH 1

CHAPTER IV

DISCUSSION

Tamoxifen is the most widely used ER antagonist therapy employed to treat ER+ breast cancers [16, 19]. Tamoxifen arrests cell growth leading to a cytostatic state by binding ERα and thus preventing estrogen from binding. While ERα bound by tamoxifen can still translocate into the nucleus it is unable to properly recruit co-activators required for transcription. Unfortunately, it has been widely reported that after long term use of tamoxifen cells can become resistant and overcome the cytostatic state created by tamoxifen [16, 19]. Resistant cells can evade the effects of tamoxifen and achieve ERα activation through kinase signaling pathways such as MAPK pathway [20, 22]. Our lab has shown that naringenin can inhibit these kinase signaling pathways and interact with ERα. However, the specific molecular targets responsible for the effects of naringenin are unknown.

The effects of naringenin and other flavonones have been studied in relation to glucose uptake, as endocrine disruptors as well as their use as a possible therapeutics in multiple cancers [6, 29, 32, 43]. It has been reported that naringenin can bind/interact with the ER [42, 43]. It has also been suggested that naringenin can affect kinase signaling pathways. However, the primary targets of naringenin have not been identified.

Our studies suggest that all of the effects observed in naringenin treated cells cannot be fully explained by the inhibition of ERK1/2 phosphorylation. Therefore, this suggests that naringenin targets other proteins to elicit its effects on decreased cell viability. Although our data shows that both naringenin and U0126 decrease cell viability (Fig. 4) and induce apoptosis (Fig. 5 and 6) to a similar extent, when used in combination there is a greater decrease that appears to be additive. Our results do not show a significant change in cell density (approximate 50% decrease) at 30 h as shown in previous reports [52]. However, this difference could be a result of our studies using Tam-R MCF-7 cells compared to MCF-7 cells used in previous studies.

Furthermore, when phosphorylated and total ERK were analyzed, naringenin was a weaker inhibitor of phosphorylation and expression than U0126 (Fig. 2). Our results showed a 40-50% decrease in phoshorylated ERK1/2 in naringenin treated cells (Fig. 2B) which is comparable to previous studies using MCF-7 breast cancer cells [43]. Our data suggests that naringenin and U0126 could be eliciting these effects through different mechanisms or by naringenin interacting with different target proteins. Naringenin could be targeting something further upstream from MEK or another protein involved in the regulation of this pathway. This would result in our observed additive effect in the combination treatments.

Since it has been reported that naringenin interacts with the ER and it has previously been shown that naringenin changes the localization pattern of the ER, we tested U0126 to see if naringenin was eliciting this change through direct interaction or through a kinase pathway. Our data demonstrated that U0126 did not

alter the ER localization pattern when compared to the untreated cells. Therefore, naringenin could be directly interacting with the ER to prevent cytoplasmic ER from entering the nucleus or it could be targeting proteins involved in ER localization and regulation. This effect is specific to naringenin and cannot be explained by inhibiting ERK1/2 expression and/or phosphorylation.

Our studies show that in the absence of estrogen, naringenin is still able to inhibit ERK1/2 phosphorylation and change ERα localization. This suggests that the effects of naringenin are not dependent on estrogen. Futhermore, the inhibition of ERK1/2 phosphorylation does not explain the effects on cell viability nor the change in ERα localization. Therefore, we focused our studies on identifying other molecular targets of naringenin. Our screen of phage display libraries identified amino acid sequences that interact with naringenin. An analysis of these sequences identified various families of proteins including known targets of naringenin such as cytochrome P450s and ER (Table 2). However, there was an unexpected abundance of proteins related to the ubiquitin pathway such as E3 ligases (Table 2). This suggests that naringenin could potentially be targeting E3 ubiquitin ligases and altering their activity. E3 ligases are enzymes involved in the ubiquitin system that ubiquitinate proteins for degradation thus effecting cell signaling pathways involved in cell proliferation and survival [37–39, 53]. Therefore, if naringenin is effecting E3 ligase activity this could lead to changes in protein expression of factors such as p53, cyclins, caspases, and membrane receptors involved in cell proliferation and viability [39, 47, 54]. Naringenin could also be utilizing the E3 ligase system to

regulate ERα. The E3 ligase UBR5 was identified as a putative target of naringenin (Table 2) and UBR5 has been reported to regulate ERα protein levels as well as affect ERα target genes and estrogen-mediated cell proliferation in breast cancer cells [51].

Studies have shown that naringenin affects multiple processes including metabolism, cell proliferation, apoptosis, and glucose uptake [6, 28–30, 52]. Furthermore, our studies in tamoxifen resistant breast cancer cells, naringenin has been shown to affect the MAPK pathway, ERα, cell proliferation, cell viability, and apoptosis. Collectively, this suggests that the effects of naringenin could be the result of naringenin targeting a more global protein such as E3 ligases which leads to multiple effects. Overall, our studies open the door for further investigation into the use of naringenin in the treatment of tamoxifen resistant, ER+ breast cancers. Further research is needed to verify the significance of the identified proteins as targets of naringenin. Also future studies will need to determine how naringenin is interacting with E3 ligases, how and if that further inhibits ERα function and localization, and if E3 ligase interaction is also related to the decrease in MAPK signaling.

Our studies suggest that naringenin could be a possible therapeutic to target E3 ligases that are overexpressed or uncontrolled in cancer cells. Many therapies target a specific protein to inhibit one pathway such as tamoxifen which directly binds the ER. While treatments like tamoxifen are effective, cells can become resistant because the targeted pathway can be by-passed with other signaling

pathways. However, our data suggests naringenin is able to affect multiple pathways without completely inhibiting them. This partial inhibition may suggest that naringenin has a low affinity for the target protein which would be desirable for treatment purposes. Partial inhibition would still allow essential pathways to function. This indicates that naringenin has the potential to be an effective therapy especially if used in combination with other drugs such as tamoxifen.

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